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**SOLUBLE PROTEINS AND LIPOPROTEINS
OF LEGUMES IN RELATION TO BLOAT**

BY

LOREN M. ROMMANN

**A thesis submitted
in partial fulfillment of the requirements for the
degree Doctor of Philosophy, Major in
Agronomy, South Dakota
State University**

1970

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SOLUBLE PROTEINS AND LIPOPROTEINS
OF LEGUMES IN RELATION TO BLOAT

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGMENTS

I wish to express my sincere appreciation to:

The U. S. Health, Education and Welfare Department; the South Dakota State University Plant Science Department; and the Northern Grain Insects Research Laboratory for their financial support and use of facilities and equipment.

Dr. R. A. Moore, major adviser, for his guidance and confidence in me.

Dr. E. D. Gerloff, thesis adviser, for his patience and counseling during the course of this research.

Dr. M. D. Rumbaugh, for some of the legume samples and data used in this research.

The other members of my graduate committee, Drs. L. O. Fine, D. J. Holden, G. A. Meyers, and B. L. Miller for their participation and encouragement throughout the program.

Mrs. Dan Gee, laboratory technician, for her assistance with laboratory procedures.

Dr. Paul Guss, biochemist, NGIRL, for conducting the thin layer chromatography reported in this work.

Jay Leibel, for his conscientious care of the plants during my absence.

My wife, Joyce, and our children, Brenda and Ann, for their patience when I was not patient and for their understanding during periods of stress.

Loren M. Rommann

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Abstract

Loren M. Rommann

Under the direction of Drs. E. D. Gerloff and R. A. Moore

Percent Fraction 1 protein, total soluble protein, and the lipoproteins of birdsfoot trefoil, white clover, and four varieties of alfalfa (Teton, Travois, Vernal, Ladak) were investigated in relation to ruminant bloat using polyacrylamide gel electrophoresis. The plants were grown in a growth chamber under two daylengths and harvested at the bloom, bud, and prebud stages of growth.

No statistical difference in percent Fraction 1 protein, measured as a percentage of the total soluble protein, could be detected among birdsfoot trefoil and three varieties of alfalfa. Both birdsfoot trefoil and Teton alfalfa were significantly lower in percent Fraction 1 protein than Vernal alfalfa and white clover. Minor differences in percent Fraction 1 protein due to daylength or stage of growth and their interactions do not appear sufficiently large to be a primary factor in the etiology of bloat.

Statistically significant differences in the amount of total soluble protein were shown among varieties or species. Both Teton alfalfa and white clover were

significantly lower than Vernal alfalfa. Birdsfoot trefoil was significantly lower than all other species.

Each species, variety, and clone tested showed a qualitatively different, dark staining, lipoprotein pattern with the exception of birdsfoot trefoil. These staining patterns were not affected by the stage of growth nor by the length of day. Definite quantitative differences were observed among six clones of Vernal alfalfa. A possible relationship between the amount of lipoproteins in legumes and the etiology of bloat is discussed.

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INTRODUCTION

Ruminant bloat is a non-infectious disease characterized by distention of the reticulo-rumen. It is found in many parts of the world and was described as early as the first century A.D. (Dougherty, 1966).

Lindhahl et al. (1957) quote an economic loss in the United States of at least \$40,000,000 annually from cattle deaths due to bloat. The time needed to treat bloated animals, cost of medications, decreased animal vigor in non-lethal cases, and possible loss of feed efficiency because of the fear of pasturing certain plant species each directly or indirectly add to the economic loss from ruminant bloat.

Alfalfa-grass mixtures for pasturing are currently being emphasized at the South Dakota Pasture Research Center. The fear of ruminant bloat is undoubtedly keeping many people from adopting this type of pasture. If an alfalfa with low bloating potential could be developed, pasture improvement might be greatly accelerated.

Most researchers agree that bloat is due to an excess accumulation of stable foam in the rumen. Identifying the agents which cause this stable foam continues to be an elusive problem.

Recently, a correlation of .97 and .99 was shown between "Fraction 1" protein in alfalfa and the severity of

bloat in sheep and cattle, respectively (Stifel et al., 1968).

Plant maturity and varietal differences in alfalfa may also be important as Lahontan was reported to contain 15% more Fraction 1 protein than Rhizoma with the largest amounts in the bud stage of growth. Increased bloat severity after a rain and decreased bloat during hot weather has also been observed (Brown et al., 1957).

The objectives of this research were to determine the effects of day length and stage of plant growth on the amount of Fraction 1 protein and to study other plant constituents related to bloat using a controlled environment with three species of legumes ranging from a non-bloating legume to legumes with high bloat potential.

REVIEW OF LITERATURE

Many plant constituents have been blamed for ruminant bloat by various researchers. Cole and Boda (1960) provide a comprehensive review of bloat up to 1960. Jacobson (1967) and Bartley (1967) summarize more recent research results. Therefore, only the most pertinent substances will be discussed in this review.

Most researchers now agree that bloat is caused by excessive formation of a stable foam in the rumen coupled with the inability of the animal to eructate normally. The evolution of large amounts of gases is a normal process associated with fermentation in the rumen. Researchers have therefore looked for the components in plants, such as certain legumes, which produce a higher or lower incidence of bloat.

Saponins

Jacobson et al. (1957) found that small amounts of saponin in the presence of glucose could aid greatly in the formation of a stable froth. Saponin digesting bacteria that produce large amounts of slime were isolated by Gutierrez, Davis, and Lindahl (1958) from the rumen contents of animals fed fresh alfalfa. These bacteria were apparently responsible for froth formation. However, Gutierrez and Davis (1962) later reported considerable

variation in the ability of the bacteria to produce slime and concluded that saponins were not the primary cause of bloat but felt they could be a contributing factor.

The maximum strength of saponin foam was also found to be at pH 4.5 to 5.0 (Mangan, 1959). Rumen liquor from animals fed a legume with a high bloating potential will show maximum foam strength at pH 5.4 to 5.7, somewhat higher than that for saponins.

Pectins

Conrad et al. (1958) wrote of a possible relationship between pectic substances and fiber to gas and foam production in the rumen. Gupta and Nichols (1962) demonstrated that adding pectin methylesterase (PME) to ruminal fluid greatly increased the viscosity, a factor in foam stability, and that PME is found in plants and rumen fluid. Aqueous extracts of fresh alfalfa contained demonstrable PME activity whereas the expressed juices of the plant did not. The role of pectins in ruminant bloat is still under investigation.

Proteins

Wildman and Bonner (1947) isolated an electrophoretically homogenous protein from spinach leaves which they designated as "Fraction 1" protein. This is a relatively

large protein with an estimated molecular weight of 500,000 to 600,000. This fraction contained 70 to 80% of the total cytoplasmic proteins and showed phosphatase enzymatic activity. The remaining cytoplasmic protein, "Fraction 2," was found to have several enzymatic properties.

Experiments with plants representing five different plant families showed that 23 to 50% of the cytoplasmic protein is Fraction 1 (Singer et al., 1952). The electrophoretic and ultracentrifugal spectrums were strikingly similar among species. A weakly acid media (pH 6.0) would precipitate Fraction 1 protein.

It was concluded by Dorner, Kahn, and Wildman (1958) that Fraction 1 protein is ubiquitous among higher plants. Several researchers have observed that much of the soluble protein in plants is located within the chloroplasts (Granick, 1938; Lyttleton and Ts'o, 1958; Zucker and Stinson, 1962; Smillie, 1963).

Lyttleton (1956) isolated a monodisperse protein from white clover with a molecular weight of about 600,000 which was similar to a type of protein he found in monocotyledonous plants. Boda et al. (1957) suggested that soluble protein in plants may be an important factor in bloat from legumes because of moderate bloat produced when fresh egg white was administered intra-ruminally to animals on a ground alfalfa ration. Analysis of the foam from bloated

animals by Bartley and Bassette (1961) showed the composition to be 63.3% protein (N X 6.25), 1.5% ether extract, 17.0% carbohydrates, 18.2 ash, and no crude fiber.

Maceration of the plant cells by the chewing action of animals was found to release the soluble proteins almost twice as fast as the foam inhibiting materials would be released (Reid, 1958). Additional support was given to the "soluble protein theory" by Boda (1958) when he observed that the incidence of bloat was reduced when alfalfa was dehydrated prior to feeding, possibly because of the denaturation of the soluble proteins.

McArthur and Miltmore (1964), using agar gel chromatography, found indications of an apparently homogeneous protein with a sedimentation constant of 18 Svedberg units (18-S protein or Fraction 1 protein) and believed this to be the cause of bloat. They proposed a mode of action which was based on the surface denaturation of proteins to form a stable foam. This is consistent with the work of Cumper (1953) who reported that only surface denatured protein is effective in stabilizing a foam bubble, noting that the stability is influenced by the viscosity of the foaming liquid.

McArthur and Miltmore (1966) also reported differences in the amount of 18-S protein between bloating and non-bloating forages. These differences ranged from about 4.5%

for the bloating forages to less than 1.0% for nonbloating forages. The 18-S protein in alfalfa increased to a maximum at the bud stage of growth and then decreased to a constant level. Varietal differences were reported with a 15% increase in 18-S protein in Lahontan over Rhizoma.

Synhorst, Wilsie, and Worthington (1963) compared samples of alfalfa and birdsfoot trefoil first collected when the plants were six inches high after the first cutting. Sampling was continued for 26 consecutive days with analyses conducted for Kjeldahl N, soluble protein (in pH 6.5 phosphate buffer and measured as trichloroacetic acid precipitate), and foam according to the method of Pressey et al. (1963). The first two measurements, reported on a percent dry weight basis, showed the Kjeldahl N content of alfalfa declining from 5.0% to 2.5%. They observed the soluble protein content of alfalfa to reach a maximum of 12% near the middle of the test period which corresponds to the time of maximum foam generation. The soluble protein subsequently declined to 9.0% near the conclusion of the experiment. Maximum soluble protein in birdsfoot trefoil was reported at the beginning of the test which also corresponds to the highest foam generation period. Soluble protein then decreased to a trace for the remainder of the test.

A direct relationship of total and soluble alfalfa leaf chloroplast proteins and the severity of bloat in cattle was reported by Stifel et al. (1968) with the closest correlation existing between Fraction 1 protein and bloat.

Jones and Lyttleton (1969) used polyacrylamide gel electrophoresis to identify the proteins in various aliquots as they were collected from a Sephadex G-50 column. The protein extraction procedure (organic solvents) was the same as that used by Stifel et al. (1968). These researchers showed that what Stifel called Fraction 2 protein was actually pigmented material containing very little protein although exhibiting relatively high absorbance at 280 mu. Fraction 2 protein was found in the trailing edge of the "peak" previously identified as Fraction 1 protein. Both protein fractions were partially removed from solution by exhaustive foaming which led to the conclusion that both Fraction 1 and Fraction 2 protein contribute to the foams produced in ruminant bloat.

Sephadex G-50 is not designed to discriminate among proteins with molecular weights greater than 75,000 and, according to Jones and Lyttleton (1969), the organic solvent method of extraction used by Stifel et al. (1968) yielded negligible amounts of Fraction 1 protein from alfalfa leaf extracts at their laboratory.

The Sephadex chromatography results of Jones and Lyttleton (1969) agree with the previously reported data of Mendiola and Akazawa (1964). They were unable to purify Fraction 1 protein with Sephadex G-75 chromatography and observed four or five peaks when aliquots from the "Fraction 1" peak of Sephadex G-75 separation were applied to Sephadex G-200. This work was conducted with rice proteins. Steer et al. (1968) also used a two stage separation procedure, Sephadex G-100 and Sephadex G-200, before they could obtain purified Fraction 1 protein from Avena sativa L.

Recently McArthur and Miltmore (1969) observed that severe bloat occurred only at pH values of 5.2 to 6.0 but that a low rumen pH alone does not produce bloat. The pH of the rumen contents during bloating conditions was near the isoelectric point of 18-S protein and the foam consisted of larger bubbles in the less severe cases of bloat than in the more severe cases.

Alfalfa and Ladino clover produced lower rumen pH values than did orchardgrass. Ladino clover produced more bloat than alfalfa.

Lipids

Researchers have been interested in the relationships of lipids to bloat because of the known antifoaming properties of some fats and oils. Reid (1959) tested numerous

compounds for their efficacy in the treatment of bloat and found a wide variety of fats and oils, some detergents, and other synthetic surface active substances were effective. Other detergents and all silicone preparations were valueless for bloat treatment. The prevention of bloat could be accomplished by drenching each cow with 50 ml of oil before feeding bloat provoking material.

Another preventative was to spray oil on a bloat provoking pasture so that each cow would consume from 90 g to 180 g of oil per day. However, he noted that the normal dietary intake of plant lipids for a lactating dairy cow is 300 g to 600 g per day when grazing bloat provoking pastures which indicates that crude lipid as determined by the usual methods is not a means of predicting bloating or nonbloating conditions. Red clover has a crude lipid content of 4% to 5% but this is not correlated with the incidence of bloat.

Reid (1959) also recognized that the chewing action of an animal releases plant lipids less efficiently than it releases the water soluble compounds. He felt that the balance of foaming and antifoaming agents or the availability of antifoaming agents within the rumen might be factors in the occurrence of bloat. Many fats and fatty acids are good antifoaming agents but there is little knowledge as to how or why they work.

The sulpholipid of red clover was demonstrated by Russell and Bailey (1966) to be effective in reducing the surface tension of water by about 50% at a concentration of 100 µg/ml but they doubted that the animal intake could be large enough to prevent bloat.

Wright (1961) found that rumen micro-organisms can hydrolyze different lipids at varying rates. Penicillin or terramycin reduced this activity which suggests that bacteria may be responsible for the lipolysis and a possible factor in bloat.

The feeding of compounds for the prevention of bloat has been investigated repeatedly. These include methyl silicone (Hugate et al., 1955), soybean oil, lard oil, lecithin, penicillin, n-decyl alcohol (Johnson et al., 1958), peanut oil, emulsified tallow, cream, polymerized dimethyl silicone (Reid, 1958), and alkyl aryl sulphonates, detergents (Nichols et al., 1957). Varying degrees of success have been noted with these compounds but Bartley et al. (1965), have used poloxalene with promising results.

Another area of interest is the association of lipids with other molecules within the plant. Bailey (1964) showed that galactolipids were rapidly released and degraded in the rumen but he could find no correlation between levels of lipid bound sugars in plants and the incidence of bloat. Sulfolipids and lysosulfolipids were reported by Shibuya,

Maruo, and Benson (1965) to be localized in the lipoproteins of spinach chloroplasts. Benson and Maruo (1958) also found that chloroplast phospholipids accounted for almost 50% of the total lipid phosphorous in sweetclover and barley with barley containing 22% more lecithin than sweetclover.

A review of the literature by Lichtenthaler and Park (1963) led them to conclude that spinach chloroplast lamellae consist of 48% protein and 52% lipid but no inferences were given as to the structural relationships.

An inverse relationship between the amount of chloroplast lipids and bloat was reported by Stifel et al. (1968). With electron microscopy, they observed numerous large, osmiophilic lipid granules in the chloroplasts from non-bloat alfalfa samples. The number and size of the lipid granules was highly correlated with changes in the amount of chloroplast lipids and bloat. They suggested that a stable foam was produced by an imbalance of protein and lipid chloroplast components which are specifically located within the stroma.

Lipids, per se, can hardly be considered the reason for the presence or absence of bloat as birdsfoot trefoil, a nonbloating legume, has a lower lipid content than white clover and alfalfa is lower than birdsfoot trefoil (Smith, 1964).

Some of the literature concerning the role of lipids in bloat is contradictory. Fraser (1961) found no differences in the lipid content of rumen liquor between bloating and nonbloating animals and no marked differences in the lipid content of samples from the same pasture on bloat and nonbloat days. Ryegrass contained a higher lipid content than either red or white clover. Emery et al. (1960) had previously reported that the lipid and nitrogenous components of rumen liquor seemed to be higher from bloated cows.

Wallace (Rep. 5th Conf. Rumen Function, Maryland, p. 24), as quoted by Wright (1961), stated that the surface coating of bloat foam bubbles was rich in lipids (12 to 24%). Laby and Weenink (1966) analyzed rumen foam from a bloated cow and reported 24.4% lipid and 30.5% protein. They also observed "yellow bubbles" occurring about 100 times more frequently in bloat samples than in nonbloat samples. These yellow bubbles contained more than 32% lipid. The lipid values reported here greatly differ from the 1.5% lipid content observed by Bartley and Bassett (1961).

Much of the research on ruminant bloat during the past decade has incriminated legume proteins as being the primary factor in the etiology of bloat. The research reported in this thesis was inspired by this avenue of reasoning.

MATERIALS AND METHODS

Experimental Conditions

Four varieties of alfalfa (Medicago sativa L.), one of birdsfoot trefoil (Lotus corniculatus L. 'Empire'), and one of white clover (Trifolium repens L.), were used in this study. The varieties 'Teton' and 'Travois' represented pasture type alfalfas whereas 'Vernal' and 'Ladak' represented hay type alfalfas. Birdsfoot trefoil represented a nonbloating legume and white clover is known to be a legume that can cause bloat in ruminants.

Two clones of each variety or species were selected from established stands on the South Dakota State University Agronomy Farm and vegetatively propagated in five-inch top diameter clay pots using a 2:1 soil-sand mixture as the potting medium. A sand filled four-inch plastic pot was placed under each clay pot to serve as a basin for excess water. Hoaglands nutrient solution was added to the soil at the rate of 25 ml per pot every two weeks. The plants were watered with distilled water whenever necessary. This varied from one watering every two days to watering each day, depending upon the size of the plants and the growth chamber conditions.

The experimental plants were in a growth chamber which furnished approximately 1200 foot candles of light measured

at the top of the pots. Cool white fluorescent bulbs supplemented by twelve 75 watt incandescent bulbs constituted the light source. The temperature was set at $26\text{ C} \pm 2$ during the light period and $17\text{ C} \pm 2$ during the period of darkness. Malathion (00,Dimethyl S-1,2-di(ethoxycarbonyl) ethyl phosphorodithioate) and Dibrom (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate) were used to control insect pests.

The plants were arranged in a randomized complete block design with four replications. Treatments were fixed and imposed as a 2 X 3 factorial experiment using daylength and the stage of plant growth as the factors, respectively. Statistical analyses were conducted according to methods described by Steel and Torrie (1960).

Daylengths of 16 and 20 hours were used and each plant harvested in the prebud, bud, and early bloom stages of growth. Plants which had reached the desired stage of growth were cut near the crown and the cut ends immersed in distilled water. A 0.5 gram (fresh weight) representative sample of leaflets was stored in a freezer at -30 C until the soluble proteins were extracted.

Protein Extraction Procedure

Soluble proteins were extracted from the leaf samples according to the method used by Gerloff, Stahmann, and Smith (1967) using a pH 8.0 buffer containing 0.1 M tris

(Hydroxymethyl) Aminomethane, 12.5% sucrose, 1.0% ascorbic acid, and 0.1% cysteine. A 500 mg leaflet sample was placed in a 7 ml stainless steel extraction cylinder, 5 ml of extraction buffer added and any trapped air expressed with a spatula. Small stainless steel balls of variable size were added until a convex meniscus extended above the top of the extraction cylinder. A gum rubber gasket was placed on top of the cylinder and held in place with a metal cap. The capped cylinder was placed in a homogenizing apparatus which provided a rapid (1725 rpm) lateral throw of approximately 1.5 times the inside diameter of the extraction cylinder. After two minutes of operation, the liquid portion of the homogenate was quantitatively transferred to a centrifuge tube and centrifuged for 15 minutes at 12,000 X G in a Servall refrigerated centrifuge. The supernatant was brought to a volume equivalent to 25 mg of leaflets (fresh weight) per milliliter of extract. All operations were conducted at 4 C and the extracts stored at -30 C in small glass vials.

Total Soluble Protein

The microbiuret method of protein determination (Bailey, 1967) was used to assay the total soluble protein in each sample.

Proteins were precipitated by adding 1.0 ml of sample to 1.0 ml of 20% trichloroacetic acid (TCA). The contents were mixed on a mechanical mixer and placed in an 85 C water bath for 15 minutes. The sample was cooled and centrifuged for 10 minutes at top speed in an International clinical centrifuge Model CL, equipped with a 221 head.

The supernatant was decanted and the precipitate washed twice with 1.0 ml of 10% TCA to remove nonprotein materials which absorb ultraviolet light at 330 m μ .

The pellet was suspended in 1.0 ml of distilled water and 3.0 ml of 4.0% NaOH added to dissolve the protein. When all the protein was dissolved, 0.2 ml of Benedicts reagent was added and the contents were mechanically stirred. The sample was allowed to react for 30 minutes and the absorbance determined at 330 m μ with a Beckman DU spectrophotometer, Model 2400 using a light beam width of 0.1 mm. Milligrams of protein per milliliter of sample were determined by comparing absorbance with a standard prepared with electrophoretically purified bovine serum albumin.

The Benedicts reagent was prepared by adding 173 g sodium citrate and 100 g sodium carbonate to warm distilled water; 17.3 g of copper sulfate were dissolved in 100 ml of water and added to the carbonate citrate solution. This mixture was brought to 1.0 liter with distilled water.

Total Protein

The Kjeldahl method of protein determination (N X 6.25) was used for the total protein analyses.

Gel Electrophoresis of Soluble Proteins

Polyacrylamide gel electrophoresis of the soluble proteins was conducted according to the method of Davis (1964). Standard 7.0% acrylamide gels were used for all electrophoretic determinations.

Pyrex tubes, 6 mm X 95 mm placed in shortened serum stoppers were used as forms for the polyacrylamide gels with 1.5 ml of the gel solution pipetted into each tube. This solution was carefully layered with water to exclude air from the gel. Polymerization progress could be followed by the disappearance of a diffuse interface between the gel solution and the water to the reappearance of a sharp, well defined interface when polymerization was complete enough for handling, usually within 30 minutes. This produced a gel about 60 mm in length.

The layering water was then drained from the tubes and 0.2 ml of large pore solution was added to each tube, layered with water, and photopolymerized about 30 minutes. Polymerization was indicated by increasing opalescence in the large pore.

Prepared gels were stored overnight at 4 C. Protein samples were thawed in cool water (about 10 minutes) and kept in an ice bath until used. After decanting the water from the polyacrylamide gels, equal amounts of protein (80 μ g), as determined by the micro-buuret protein analysis, were pipetted into each tube. This amounted to about 0.08 ml of sample per gel for the alfalfa varieties. Each tube was then filled with upper reservoir solution and placed in the rubber grommets of the upper reservoir. The gels were vertical and equidistant from the upper reservoir during electrophoresis to assure uniform electrophoresis of the protein bands. Duplicate gels were used for each experimental unit protein sample.

The bottom reservoir was filled with 500 ml of a buffer solution containing 0.6 g Tris, and 2.88 g glycine per liter. The same buffer was used for the upper reservoir with the addition of two drops of 0.001% Bromphenol blue (BPB) dye marker to show when electrophoresis should be stopped. This marker migrated ahead of the proteins and was visible throughout the electrophoretic process.

Electrophoresis was performed at 2.5 ma per gel using a constant current power supply with the lower reservoir carbon as the anode. Approximately three hours was required for the BPB marker band to approach the end of the gel. All electrophoresis procedures were conducted at 4 C.

The gels were removed at room temperature by introducing a two-inch, 23 gauge hypodermic needle between the pyrex tube and the gel at the lower end of the gel and rimming the gel while water flowed through the needle. When the end of the gel protruded from the tube, the upper end of the gel was rimmed in a similar manner which allowed the gel to slip out of the tube. The gel was immediately immersed in a test tube containing 1% (w/v) Amido Black in 7.0% acetic acid. This process immobilized the proteins and provided a method of determining the number of proteins of different sizes or varying negative electrical charges (or both) and the relative amounts of each protein. Protein staining was conducted for one hour after which the gel was placed in a 7.5 mm diameter tube constricted at one end. The tube was filled with the destaining polymer described by Davis (1964). The tube was placed on the electrophoresis apparatus using 7.0% acetic acid in the reservoirs and a current of 5 ma per tube. The unbound stain was removed during the overnight destaining period and the gels were stored in test tubes containing 7.0% acetic acid until densitometry measurements were made.

Densitometry

Semi-quantitative measurements of Fraction 1 and Fraction 2 proteins in each gel were made with a Chromascan

densitometer manufactured by Joyce, Loebel and Co., Ltd., Gateshead, England. The gel was placed in 9 mm X 105 mm glass tube filled with 7% acetic acid and secured between the light source and the photoelectric cell. A horizontal light beam 1 mm X 5 mm was used with a 40 degree wedge angle on the pen activator. Chart speed to sample gel horizontal speed ratio was 3:1. Readings were taken from the BPB marker to the top of the gel. The densitometer was equipped with an integrator to give a numerical estimate of the area under the graph peaks which was used to calculate the percentage of Fraction 1 protein as a percentage of the total soluble protein.

Sephadex Chromatography

Fraction 1 protein in the polyacrylamide gels was identified by the protein extraction procedure and subsequent Sephadex chromatography used by Stifel et al. (1968). A 1.0 X 15 cm Sephadex G-50-80 (particle size 20 to 80 u) column was used with an eluent head of 48 cm and a flow rate of 0.4 ml per minute. All Sephadex procedures were conducted at 4 C.

Aliquots from both the aqueous protein extraction method used in this experiment and nonaqueous methods of protein extraction used by Stifel et al. (1968) were collected as they were eluted through the Sephadex column.

Absorbance of each aliquot at 280 mμ was determined with a Bausch and Lomb Spectronic 505 spectrophotometer to determine those aliquots containing Fraction 1 protein.

Nonaqueously extracted proteins eluted through the Sephadex column with ion-free water produced no protein bands when subjected to polyacrylamide gel electrophoresis. Using 0.1 M Tris, 0.1% cysteine, and 5.0% sucrose, pH 8.0 as the eluent kept the proteins in a condition suitable for electrophoresis.

The aliquots containing Fraction 1 protein from each extraction method were used for gel electrophoresis. The protein bands in each gel had approximately the same relative mobility, differences being due to unequal amounts of protein. Mixtures of aliquots from the aqueous and non-aqueous extraction procedures showed one relatively large, dark staining band near the top of the gel and some minor bands lower in the gel when subjected to electrophoresis. This large band has been described as Fraction 1 protein by Jones and Lyttleton (1969), and Wilson and McCalla (1968) with polyacrylamide gel electrophoresis.

Lipid Analysis

The chloroform-methanol lipid extraction method described by Bligh and Dyer (1959) was used to extract the lipids from 0.8 ml of protein extraction sample for use in thin layer chromatography (TLC) analyses.

Neutral lipid TLC was performed according to the procedures of Guss (1969) while the TLC for polar and phospholipids was a method reported by Skipski, Peterson, and Barclay (1964). Iodine vapor was used to detect the polar lipids and phospholipid specific spray (Dittmer and Lester, 1964) was later used on the same plate.

Lipoprotein Staining

Procedures used to obtain polyacrylamide gels showing the lipoprotein patterns were identical to those used for protein analysis up to the point of staining the gels.

Lipoprotein staining was accomplished by placing each gel in 0.1% Sudan Black B-50% ethyl alcohol (w/v) solution for overnight staining (Dejmal and Brookes, 1968) with one change of staining solution.

Destaining was with several changes of 40% ethyl alcohol. The gels could then be stored in 40% ethyl alcohol or in 7% acetic acid to restore them to their original size for comparison with gels stained for proteins.

Samples from one replication from each treatment were stained for lipoproteins.

Foaming

Foaming tests with subsequent electrophoresis were conducted at room temperature by placing about 3.0 ml of

soluble protein extract in a separatory funnel and blowing nitrogen gas through the sample. The flow of gas was through a hypodermic needle and was adjusted to produce a small bubble. Foaming was continued until the foam mass reached an apparent constant volume. The remaining liquid (mother liquor) was drained from the funnel and subjected to electrophoretic analysis with duplicate gels stained for proteins and lipoproteins.

Exhaustive foaming was considered to be 15 minutes of continuous foaming with subsequent electrophoretic analysis. The foam was condensed after the mother liquor was removed and analyzed electrophoretically.

RESULTS AND DISCUSSION

Proteins

General Observations

Figure 1 shows polyacrylamide gel electrophoresis of each alfalfa variety (Teton, Travois, Vernal, Ladak), birdsfoot trefoil, and white clover. Each dark stained band indicates a separate protein. The plants were grown with a daylength of 16 hours and harvested at the bud stage of growth. The areas which will be referred to as Fraction 1 (18-S) protein and Fraction 2 proteins are identified as well as the position of the electrodes during electrophoresis. Thirteen protein bands could be visually detected in the Fraction 2 portion of alfalfa samples.

Qualitative differences can be detected among species but not among varieties of alfalfa nor between clones within a variety or species. These species differences are illustrated in Fig. 1 and in the representative densitometer tracings in Fig. 2. No qualitative differences were found due to daylength or the stage of growth at which the plants were harvested.

The differences shown among the three species tested are distinct. Birdsfoot trefoil was the most outstanding in this respect as it showed a larger area in the polyacrylamide gel between Fraction 1 and Fraction 2 protein

Fig. 1. Electrophoresis of alfalfa, birdsfoot trefoil, and white clover soluble proteins.

A-Teton alfalfa, B-Travois alfalfa, C-Vernal alfalfa, D-Ladak alfalfa, E-birdsfoot trefoil, F-white clover.

+ - position of electrodes during electrophoresis.

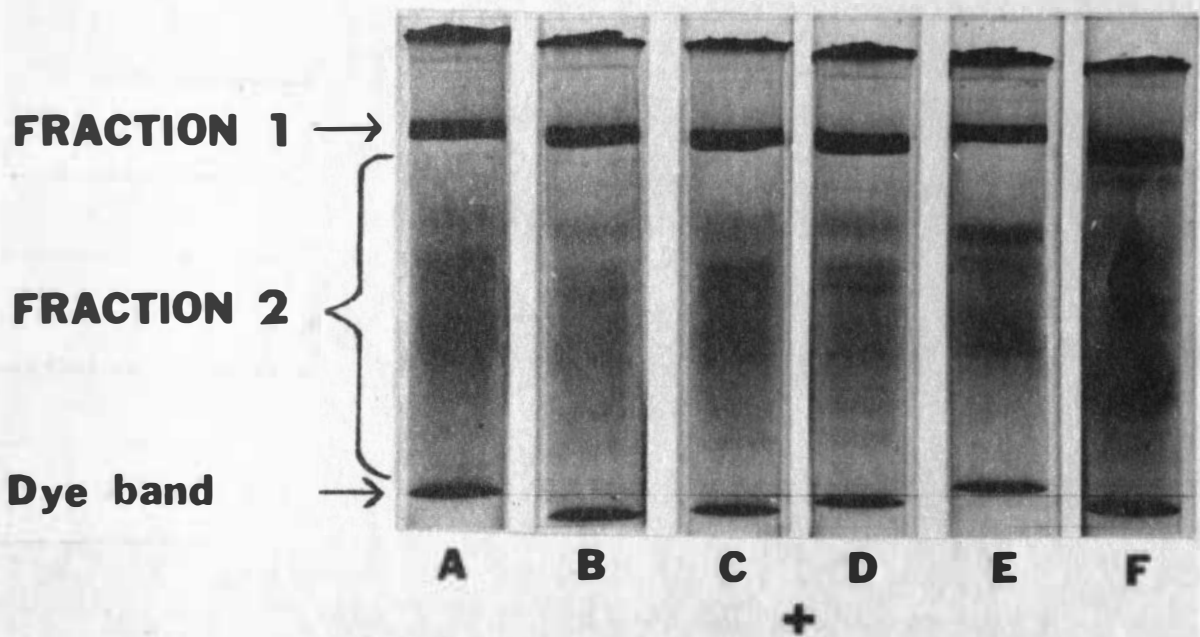


Fig. 1

Fig. 2. Densitometer tracings of alfalfa, birdsfoot trefoil, and white clover soluble proteins.

d-dye band, 2-Fraction 2 protein, 1-Fraction 1 protein, t-top of gel.

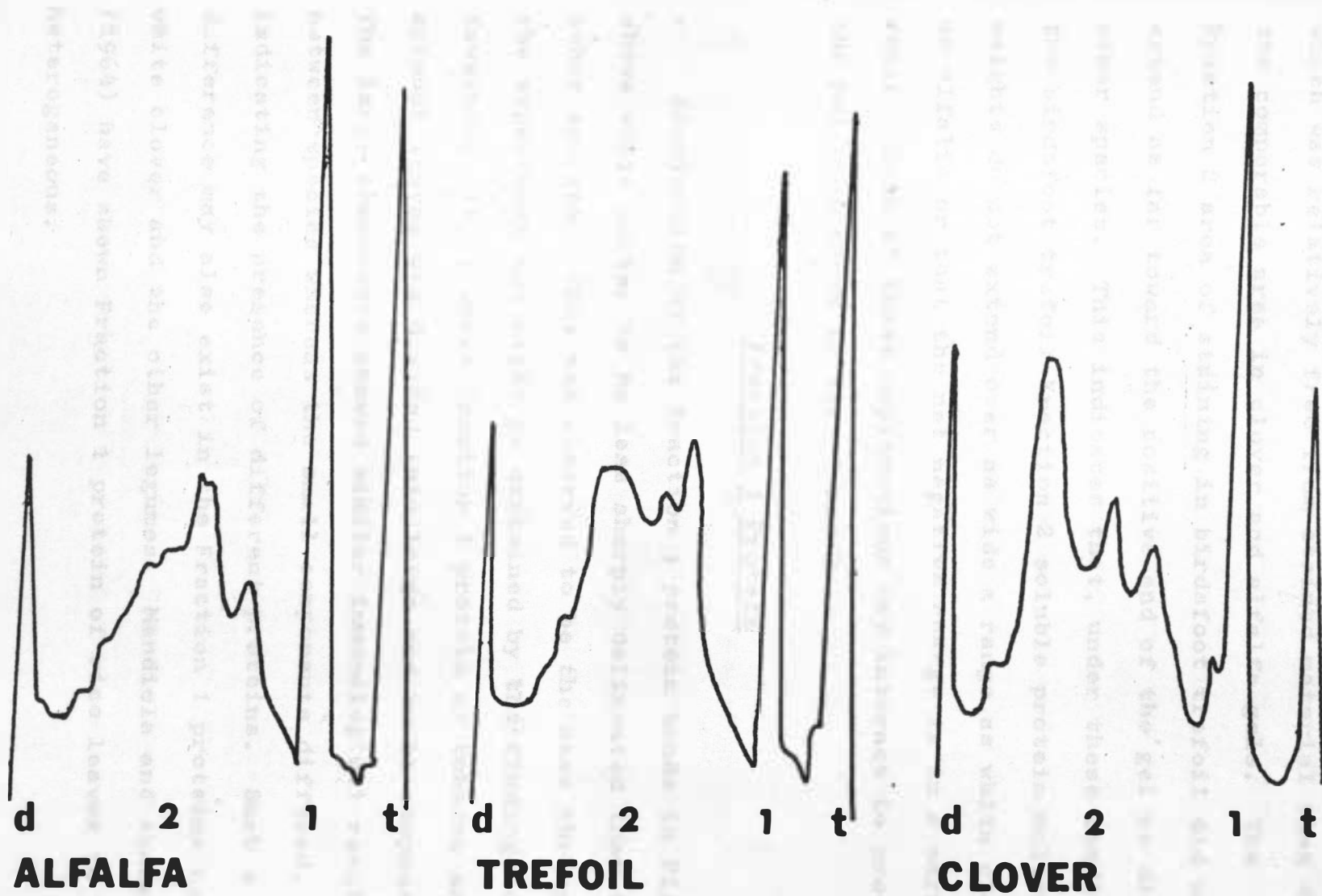


Fig. 2

which was relatively free from stained material than did the comparable area in clover and alfalfa gels. The Fraction 2 area of staining in birdsfoot trefoil did not extend as far toward the positive end of the gel as did the other species. This indicates that, under these conditions, the birdsfoot trefoil Fraction 2 soluble protein molecular weights do not extend over as wide a range as white clover or alfalfa or that the net negative charge is in a narrower range. Both of these explanations may interact to produce the patterns shown in Fig. 1 and 2.

Fraction 1 Protein

Examination of the Fraction 1 protein bands in Fig. 1 shows white clover to be less sharply delineated than the other species. This was observed to be the case throughout the experiment and might be explained by the findings of Kawashima (1969) where Fraction 1 protein of tobacco and spinach leaves was divided into large and small components. The large components showed similar immunological results between species whereas the small components differed, indicating the presence of different proteins. Such a difference may also exist in the Fraction 1 proteins between white clover and the other legumes. Mendiola and Akazawa (1964) have shown Fraction 1 protein of rice leaves to be heterogeneous.

The R_f values for Fraction 1 protein were similar among white clover, birdsfoot trefoil and alfalfa in this experiment.

Analysis of variance for percent Fraction 1 protein, measured as a percentage of the total soluble protein, identified only two sources of statistically significant variation when all test plants were included (Table 1). These sources were varieties or species and the variety or species X daylength X stage of growth X clone interaction.

When analyzing data such as these among different species of plants, the analyses can be misleading and one could expect to find significant differences among treatments and their interactions. More meaningful information is shown in Table 2 which gives the analysis of variance using only the data from the alfalfa varieties. This shows clones and the variety X stage of growth X clone interaction to be statistically significant sources of variation. No difference was found among varieties until the test was conducted at the $P = 0.10$ level.

Table 3 shows the varietal means for percent Fraction 1 protein increasing 2.8% from the lowest to the highest variety or species. Teton alfalfa has the lowest value with 29.9% and Vernal alfalfa the highest value with 32.7%

Table 1. Analysis of variance of percent Fraction 1 protein and total soluble protein for birdsfoot trefoil, white clover and four varieties of alfalfa.

Source ¹	Percent Fraction 1 Protein		Mg of Protein per ml of sample	
	<u>DF</u>	<u>MS</u>	<u>DF</u>	<u>MS</u>
Total	575		287	
R	3	594.88	3	.0185
V	5	124.08*	5	2.4848
RV	15	36.59	15	.0114
D	1	44.28	1	.2048
RD	3	109.77	3	.0287
VD	5	11.01	5	.0589*
RVD	15	13.96	15	.0177
S	2	340.10	2	.0076
RS	6	174.05	6	.0248
VS	10	30.04	10	.0180
RVS	30	26.29	30	.0112
DS	2	74.72	2	.0365
RDS	6	108.83	6	.0209
VDS	10	11.17	10	.0143
RVDS	30	23.84	30	.0229
C	1	111.92	1	.0523
RC	3	13.15	3	.0353
VC	5	15.83	5	.1161**
RVC	15	20.08	15	.0249
DC	1	37.67	1	.0024
RDC	3	9.20	3	.0049
VDC	5	7.51	5	.0041
RVDC	15	18.02	15	.0212
SC	2	11.99	2	.0079
RSC	6	30.45	6	.0123
VSC	10	19.98	10	.0103
RVSC	30	13.74	30	.0166
DSC	2	10.27	2	.0251
RDSC	6	19.47	6	.0087
VDSC	10	11.43*	10	.0215
Residual	318	5.20	30	.0136

¹R=replications; V=variety or species; D=daylength;
S=stage of growth; C=clone

*Statistically significant at $P = 0.05$

**Statistically significant at $P = 0.01$

Table 2. Analysis of variance of percent Fraction 1 protein and total soluble protein for alfalfa varieties.

Source ¹	Percent Fraction 1 Protein		Mg of Protein per ml of sample	
	DF	MS	DF	MS
Total	383		191	
R	3	340.04	3	.01298
V	3	127.89	3	.07593*
RV	9	37.76	9	.01464
D	1	19.04	1	.28060**
RD	3	95.51	3	.00792
VD	3	2.77	3	.01586
RVD	9	10.95	9	.02124
S	2	167.04	2	.00054
RS	6	89.01	6	.02374
VS	6	32.49	6	.01571
RVS	18	16.22	18	.01270
DS	2	27.02	2	.01799
RDS	6	123.85	6	.02398
VDS	6	8.52	6	.01729
RVDS	18	15.03	18	.02965
C	1	99.33*	1	.02341
RC	3	4.62	3	.04087
VC	3	9.02	3	.12811
RVC	9	22.23	9	.03588
DC	1	36.20	1	.00241
RDC	3	8.94	3	.02292
VDC	3	6.74	3	.00642
RVDC	9	16.01	9	.01529
SC	2	12.65	2	.00249
RSC	6	22.29	6	.01644
VSC	6	29.33*	6	.00812
RVSC	18	8.66	18	.01770
DSC	2	5.60	2	.00920
RDSC	6	11.37	6	.02503
VDSC	6	17.50	6	.01741
RVDC	18	16.79	--	--
Residual	192	3.17	18	.01164

¹R=replication; V=variety; D=daylength; S=stage of growth; C=clone.

* Statistically significant at P = 0.05

** Statistically significant at P = 0.01

Table 3. Daylength and stage of growth effects on percent Fraction 1 protein.¹

Variety or Species	16 hour day			20 hour day			Clonal means	Variety or Species ²
	Clone	Bloom	Bud	Prebud	Bloom	Bud	Prebud	
Teton	I	28.9	28.5	28.4	30.0	30.8	27.9	29.9 a
	II	27.8	34.2	30.6	30.5	31.9	29.7	
Travois	I	28.9	31.1	31.3	28.6	31.5	31.1	30.9 ab
	II	32.3	31.3	30.5	31.6	32.6	30.4	
Vernal	I	32.0	32.6	30.9	33.1	33.1	34.0	32.7 b
	II	34.4	32.8	32.1	33.9	35.1	28.6	
Ladak	I	27.7	32.2	29.5	30.1	34.7	29.0	31.0 ab
	II	28.8	33.5	32.5	29.8	34.4	30.4	
Birdsfoot Trefoil	I	25.2	30.2	29.6	28.3	32.1	29.1	30.1 a
	II	26.9	32.7	30.7	32.1	33.6	30.4	
White Clover	I	30.4	33.7	32.2	33.5	33.7	32.8	32.5 b
	II	30.7	34.5	33.2	32.4	33.6	29.4	
Treatment means		29.5	32.3	31.0	31.2	33.1	30.2	
Daylength means			30.9			31.5		

¹Average of duplicate gels of each sample and across four replications.

²Variety or species means followed by the same letter do not differ at the P = 0.05 level using Duncan's multiple range test.

Fraction 1 protein. Birdsfoot trefoil, the nonbloating legume, was slightly higher than Teton alfalfa with both of these species showing statistically significant differences from Vernal alfalfa and white clover.

Percent Fraction 1 protein was found to reach a maximum at the bud stage of growth under both daylengths when the data were averaged through varieties and species (Table 3) with lower values observed at both the bloom and prebud stages of growth. This is in agreement with the findings of McArthur and Miltmore (1966).

Individually, each variety or species demonstrated a similar trend with some exceptions in Vernal alfalfa and white clover.

The differences in percent Fraction 1 protein due to varietal, species, daylength or stage of growth do not appear large enough to materially affect the incidence of bloat when one considers the total amount of feed ingested by an animal grazing these legumes. Recognition must also be made of the fact that no statistical difference in percent Fraction 1 protein could be shown among birdsfoot trefoil and three varieties of alfalfa.

Total Soluble Protein

The analysis of variance of the milligrams of soluble protein per milliliter of extraction sample is shown in

Table 1. Statistically, the variety X daylength interaction is significant and the variety X clone interaction is highly significant. When the alfalfa varieties are analyzed separately, variety becomes a statistically significant source of variation while daylength is a highly significant source of variation. The variety X clone interaction is significant if tested at the $P = 0.10$ level.

Varieties or species mean values of milligrams of solution protein per gram of fresh weight tissue (Table 4) range from 40.4 to 44.2, excluding birdsfoot trefoil. Birdsfoot trefoil shows a statistically significant lower value than all other varieties or species. White clover and Teton alfalfa are both significantly lower than Vernal alfalfa. The total soluble protein content follows a pattern similar to the total protein values reported in Table 5.

Differences in total soluble protein may have considerable importance in determining whether bloat occurs or not. The high correlations between Fraction 1 protein and bloat in sheep and cattle reported by Stifel et al. (1968) could more accurately be stated as correlations between the total soluble protein and bloat if the results of Jones and Lyttleton (1969) and this research are accepted. It is recognized that different soluble protein extraction methods were used by Stifel et al. (1968) than in this research, and this could affect the amount or type of proteins extracted.

Table 4. Daylength and stage of growth effects on total soluble protein.¹

Variety or Species	16 hour day				20 hour day			Clonal means	Variety or Species ²
	Clone	Bloom	Bud	Prebud	Bloom	Bud	Prebud		
Teton	I	39.1	38.8	38.5	38.3	42.2	36.5	38.9	40.6 a
	II	38.8	43.5	41.2	44.6	44.6	41.0	42.3	
Travois	I	37.8	44.4	42.8	45.3	43.9	47.0	43.5	43.2 ab
	II	39.0	42.7	43.0	47.3	43.1	39.7	42.8	
Vernal	I	38.3	37.4	41.6	45.8	43.6	43.0	41.7	44.2 b
	II	45.5	44.8	43.1	50.2	48.8	47.8	46.7	
Ladak	I	41.9	39.1	43.0	46.3	45.8	45.1	43.5	42.3 ab
	II	39.1	38.4	47.6	38.2	38.1	45.6	41.2	
Birdsfoot Trefoil	I	19.1	19.9	18.0	16.6	16.0	14.9	17.4	19.9 c
	II	23.2	26.2	22.5	21.1	20.9	20.9	22.5	
White Clover	I	30.6	42.6	45.1	44.1	42.5	43.7	41.4	40.4 a
	II	38.5	37.8	36.7	40.1	38.4	44.4	39.3	
Treatment means		35.9	38.0	38.6	39.8	39.0	39.1		
Daylength means			37.5			39.3			

¹Milligrams of soluble protein per gram of fresh weight tissue averaged across four replications.

²Variety or species means followed by the same letter do not differ at the P = 0.05 level using Duncan's multiple range test.

Table 5. Total protein¹ of birdsfoot trefoil, white clover, and three varieties of alfalfa.

Species or variety	Percent Protein
Birdsfoot trefoil	15.1
White Clover	23.3
Travois alfalfa	25.1
Teton alfalfa	28.0
Vernal alfalfa	28.8

¹Dry matter basis, N X 6.25 -- Average of all stages of growth.

At the start of this experiment the non-aqueous chloroplast protein extraction method of Stifel et al. (1968) was duplicated with subsequent elution through a column packed with Sephadex G-50. Absorbance at 280 mu of each 1 ml aliquot produced the graph shown in Fig. 3. Electrophoresis of aliquots from the first peak and one aliquot from the center of the second peak produced the results shown in Fig. 4. Gels A through E are aliquots from the first peak corresponding to tube numbers 3, 4, 5, 6, and 7. Gel F is the sample from tube number 18.

Fraction 1 protein increases to a maximum in gel C and then decreases in gels D and E with no Fraction 1 protein in gel F (Fig. 4). When the Fraction 2 protein of gels A through E is compared to the corresponding portion of gel F, it is demonstrated that Fraction 2 protein was eluted through the Sephadex column along with Fraction 1 protein. This is not in complete agreement with the observations of Jones and Lyttleton (1969) as they found Fraction 2 protein to be in the trailing edge of the first absorbance peak (Fig. 3) when they used gel electrophoresis for checking aliquots from a Sephadex G-50 column. This difference may be attributed to the different extraction buffer, electrophoretic technique, or chromatographic methods used in each case. The results are similar enough to support the work of Jones and Lyttleton (1969) rather than that of Stifel et al.

Fig. 3. Sephadex chromatography of alfalfa soluble proteins.

Abscissa-tube number of 1.0 ml aliquots collected
from Sephadex G-50 column.

Ordinate-absorbance at 280 mu.

Tubes 1 through 11 = Fraction 1 protein.

Tubes 13 through 25 = Fraction 2 protein.

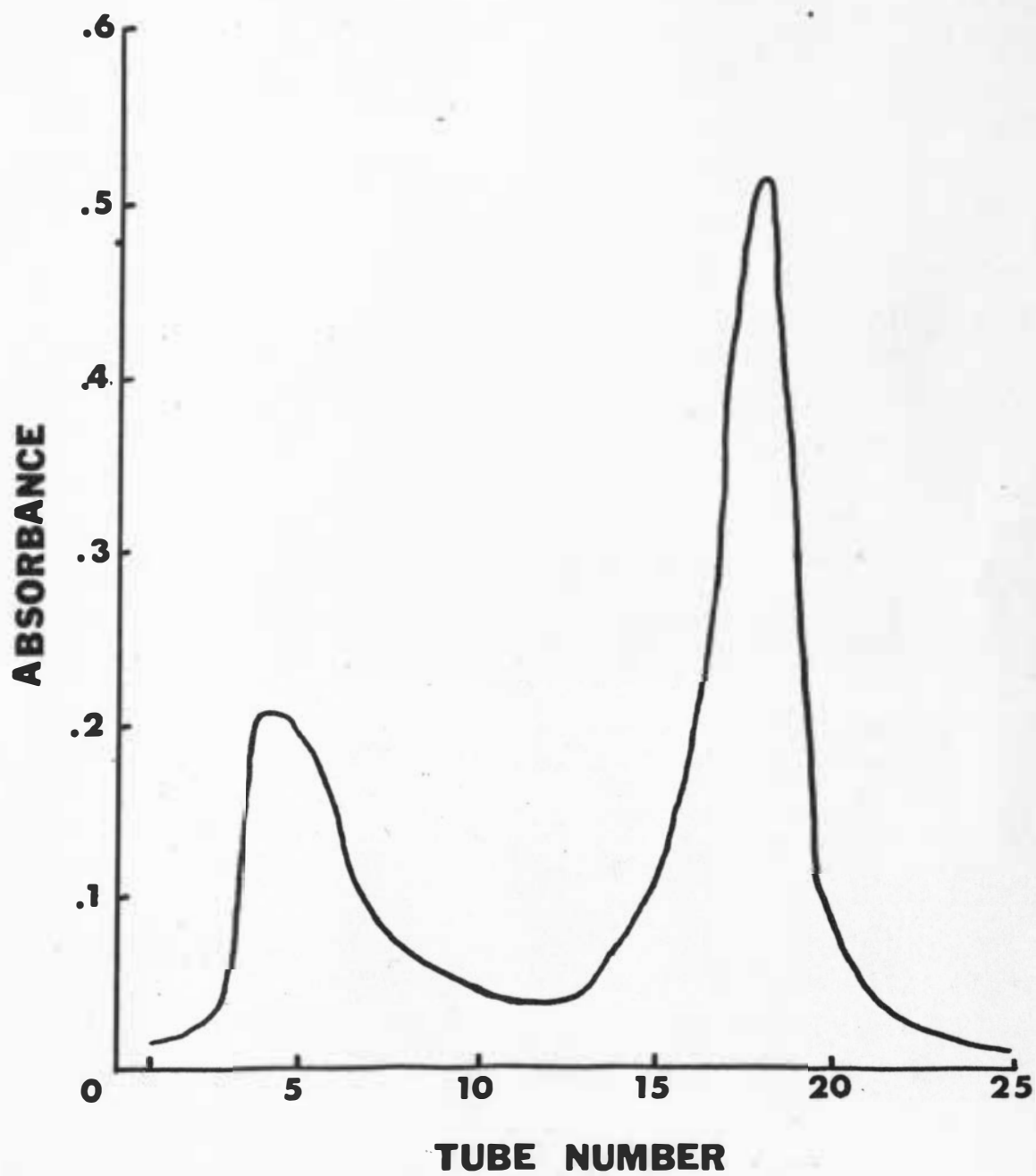


Fig. 3

Fig. 4. Electrophoretic analysis of alfalfa soluble proteins after Sephadex chromatography.

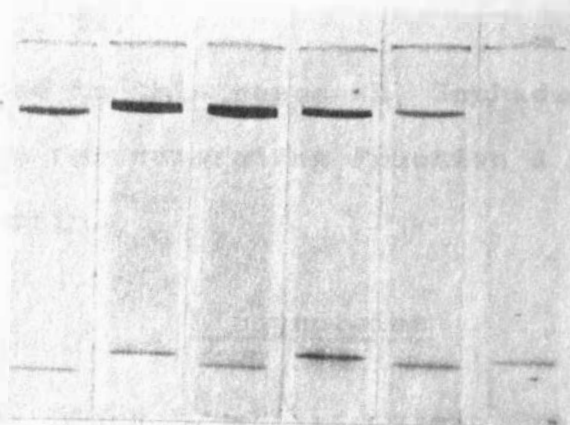
Gels A through E - Fraction 1 protein peak.
Gel F - Fraction 2 peak.

Figs. 5 and 6. Lipoprotein patterns in relation to varieties, species, clones, and treatments.

Fig. 5 - Clone 1.
Fig. 6 - Clone 2.

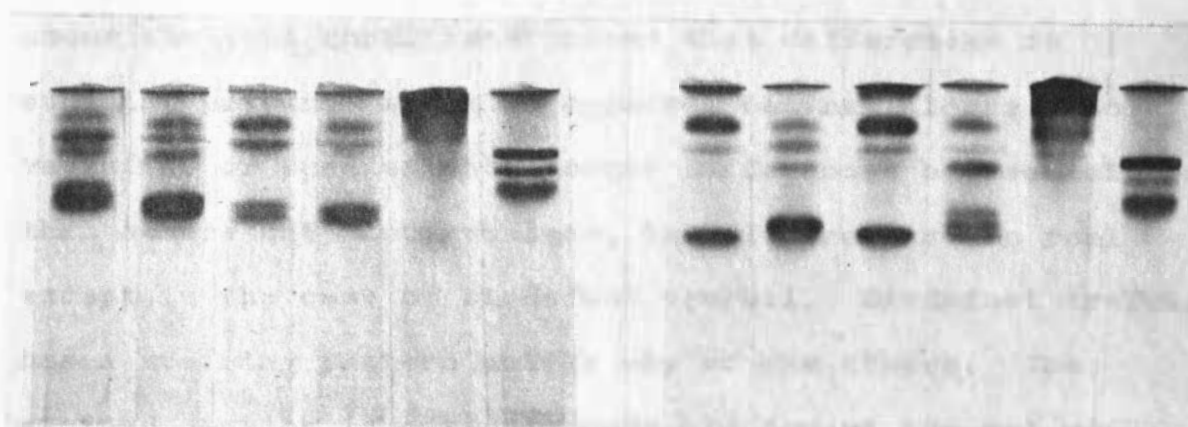
Both clones grown under a 20-hour daylength and harvested in the bud stage of growth.

A-Teton, B-Travois, C-Vernal, D-Ladak,
E-Birdsfoot trefoil, F-White clover.



A B C D E F

Fig. 4



A B C D E F

A B C D E F

Fig. 5

Fig. 6

(1968). The Fraction 2 protein is not in the second absorbance peak (Fig. 3).

As employed in this research, Sephadex G-50 is not an adequate medium for separating Fraction 1 protein from Fraction 2 protein.

Lipoproteins

When Sudan Black B, a lipid stain, was used to stain the lipoproteins, definite and distinct bands appeared in the gels (Fig. 5). Different band patterns were observed for each of the varieties of alfalfa as well as for birdsfoot trefoil and white clover.

Comparison of Fig. 5 and 6, which represent two different clones of the same varieties or species grown under the same conditions, shows that differences in staining patterns are also apparent between clones. Some varieties or species show larger differences between clones than others but, nevertheless, the differences are real except in the case of birdsfoot trefoil. Birdsfoot trefoil has a staining pattern unlike any of the others. The stained portion is entirely near the top of the gel with only a slight amount of banding.

Staining patterns did not appear to be affected by treatments in any visually detectable manner as illustrated by Fig. 7 and 8 which represent a different treatment than

Figs. 7 and 8. Lipoprotein patterns in relation to varieties, species, clones, and treatments.

Fig. 7 - Clone 1.

Fig. 8 - Clone 2.

Both clones grown under a 16-hour daylength and harvested in the bloom stage of growth.

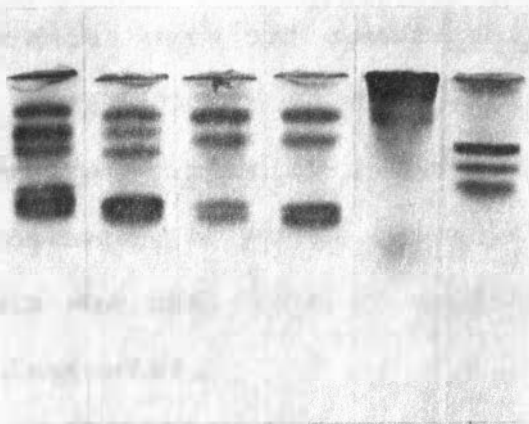
**A-Teton, B-Travois, C-Vernal, D-Ladak,
E-Birdsfoot trefoil, F-White clover.**

Figs. 9 and 10. Treatment effects on lipoprotein patterns.

Fig. 9 - Vernal alfalfa, Clone 1.

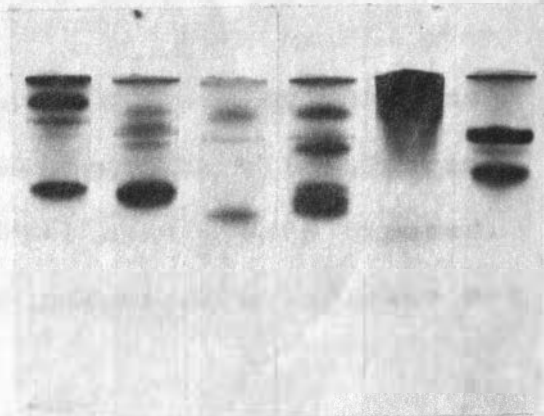
Fig. 10 - White clover, Clone 2.

**The daylength and stage of growth treatments in each figure are (left to right) 16-hour day-
bloom bud, prebud; 20 hour day- bloom, bud,
prebud.**



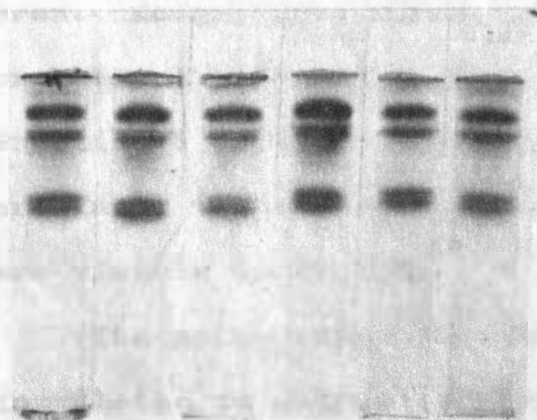
A B C D E F

Fig. 7



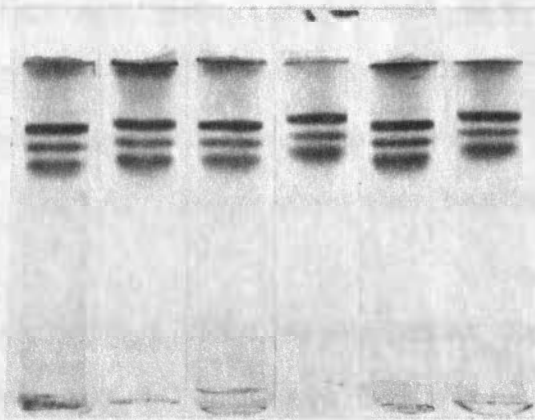
A B C D E F

Fig. 8



VERNAL 1

Fig. 9



CLOVER 1

Fig. 10

Fig. 5 and 6. Some patterns appear darker but these differences were not consistent.

Fig. 9 and 10 demonstrate how the staining patterns remained virtually identical throughout the six different treatments to which the experimental plants were exposed. This was also true of each of the varieties or species not illustrated.

The bands appearing with this staining procedure are believed to be composed of lipoproteins. This hypothesis is supported by the electrophoretic mobility of the bands and by the correspondence of the lipid bands with the protein stained bands of duplicate gels (Fig. 11). Generally, a lightly stained lipid band appeared at a position corresponding to the Fraction 1 protein band. The other lipid bands appeared either above or below the Fraction 1 protein area. Those lipid bands above the Fraction 1 proteins are probably lipids associated with proteins larger than the Fraction 1 proteins which have previously been reported as artifacts (Mendiola and Akazawa, 1964). Such protein bands are visible in Fig. 1.

The polar extraction buffer used in this experiment is formulated to extract proteins and not to extract lipids from plant tissues. This further supports the hypothesis that these stained bands are lipoproteins.

Fig. 11. Protein and lipoprotein pattern comparisons of varieties and species.

A-Teton, B-Travois, C-Vernal, D-Ladak, E-Birdsfoot trefoil, F-White clover.

Fig. 12. Alfalfa lipoproteins after elution through Sephadex G-50.

Gels A through E - Fraction 1 protein peak.
(Fig. 3)

Gel F - Fraction 2 peak.
(Fig. 3)

Fig. 13. Lipoprotein patterns of six different clones of Vernal alfalfa.

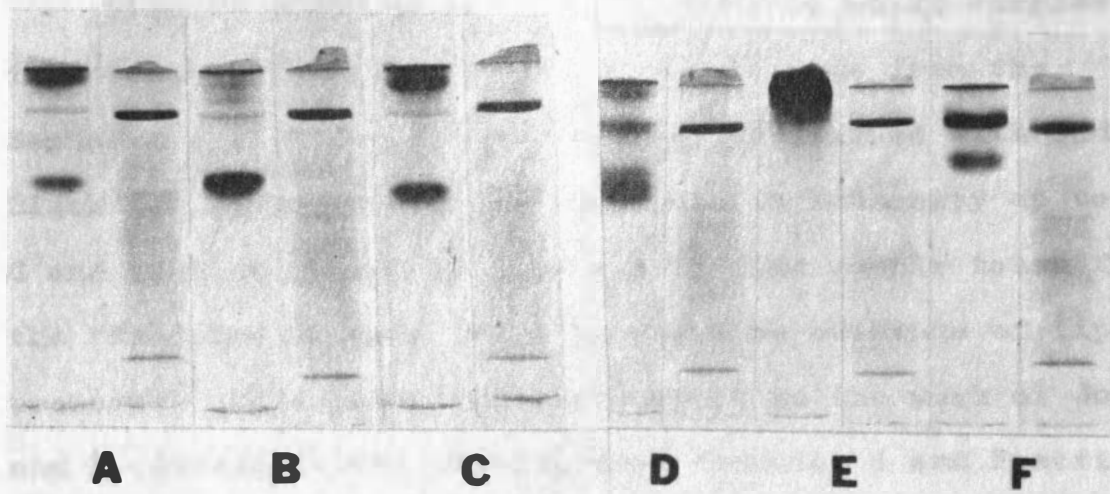


Fig. 11

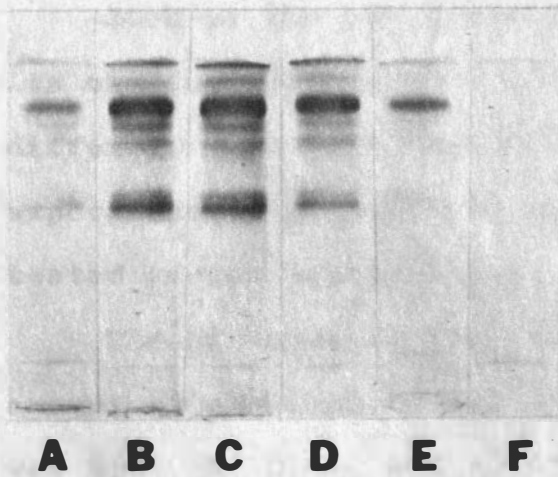


Fig. 12

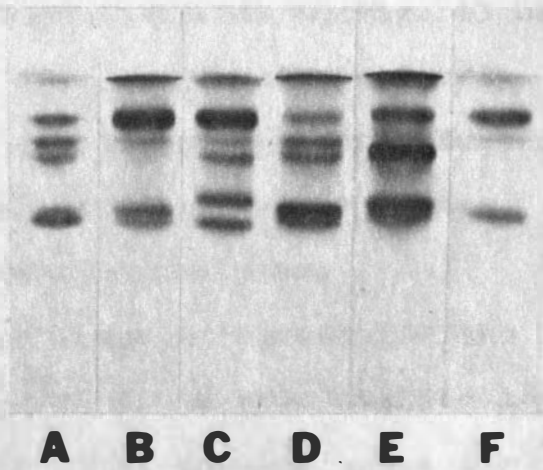


Fig. 13

Fig. 12 shows polyacrylamide gels to which samples identical to those used in Fig. 1 (aliquots from the Sephadex G-50 column) were applied and stained with Sudan Black B. Again, the bands increased in intensity up to gel C and then decreased through gel E. The sample taken from the "Fraction 2" peak (Fig. 3) shows no evidence of lipoproteins. This lends further support to the work of Jones and Lyttleton (1969) showing that Fraction 1 and Fraction 2 proteins elude together from a Sephadex G-50 column.

Samples of six different clones of Vernal alfalfa were obtained from Dr. M. D. Rumbaugh, Professor of Agronomy, South Dakota State University. These protein extraction samples were tested for lipoproteins by staining with Sudan Black B and produced the staining patterns shown in Fig. 13.

Each of the six clones of Vernal alfalfa in Fig. 13 has its own characteristic staining pattern. These patterns differ from the two clones used in the protein determination experiment (Fig. 5 and 6) making a total of eight clones tested giving eight different staining patterns.

The six gels in Fig. 13 were visually ranked for the amount of lipid staining. The order given from high to low was E, C, D, B, A, and F. The in vitro foaming value for each clone had previously been determined by the method used by Rumbaugh (1968). Reported values in cc of stable foam were D, 212; E, 185; C, 165; A, 145; B, 142; and F, 135.

Comparison of the visual ranking of lipid staining with the foam value ranking of each clone is presented in Table 6. Although the rankings do not correspond exactly, they do show that the three clones with the highest foaming values are also the three clones showing the most lipid staining.

These data then lead to the hypothesis that all plant lipids are not necessarily antifoaming agents but, when present as lipoproteins, they are the surfactants responsible for the formation of stable foam during ruminant bloat.

The polar and nonpolar portions of lipoproteins give these molecules properties that could make them effective surfactants in stable foam formation during bloat.

It is not intended to infer that the only role of plant lipids is that of lipoproteins nor that some lipids do not serve as effective antifoaming agents. The balance between lipids and lipoproteins may be important in the etiology of bloat.

Thin Layer Chromatography

To further elucidate the characteristics of the lipids present in the samples, thin layer chromatography was employed to test for neutral, polar, and phospholipids.

Table 6. Ranking of in vitro foaming value and amount of lipoprotein staining.

<u>Foam</u>		<u>Lipoprotein</u>
1	highest	2
2		3
3		1
4		5
5		4
6	lowest	6

Visual evaluation of the thin layer plates revealed no striking differences in polar lipids among varieties or species. The test for phospholipids was negative, but they may have been present in amounts too small to detect under these conditions.

Birdsfoot trefoil was found to contain at least two times the quantity of neutral lipids as any other variety or species when analyzed on an equal protein basis.

No qualitative differences in polar or neutral lipids could be detected among varieties or species.

Foaming Tests

Proteins in the samples used for the foaming tests were not appreciably denatured by the foaming process. Stifel et al. (1968) and Jones and Lyttleton (1969) both reported denaturation of proteins after foaming as evidenced by decreased ultraviolet absorbance. If the foaming in this research had denatured the proteins to any great extent, the protein and lipoprotein patterns (Fig. 14) would probably not remain the same between foamed and unfoamed samples. However, denaturation may have been prevented by the extraction buffer used.

Fig. 14. Protein and lipoprotein patterns after foaming alfalfa soluble protein extracts.

- A-Protein pattern of original sample, not foamed.
- B-Protein pattern of mother liquor after foaming.
- C-Lipoprotein pattern of mother liquor after foaming.
- D-Protein pattern of mother liquor after exhaustive foaming.
- E-Lipoprotein pattern of mother liquor after exhaustive foaming.
- F-Protein pattern of foam after exhaustive foaming.
- G-Lipoprotein pattern of foam after exhaustive foaming.

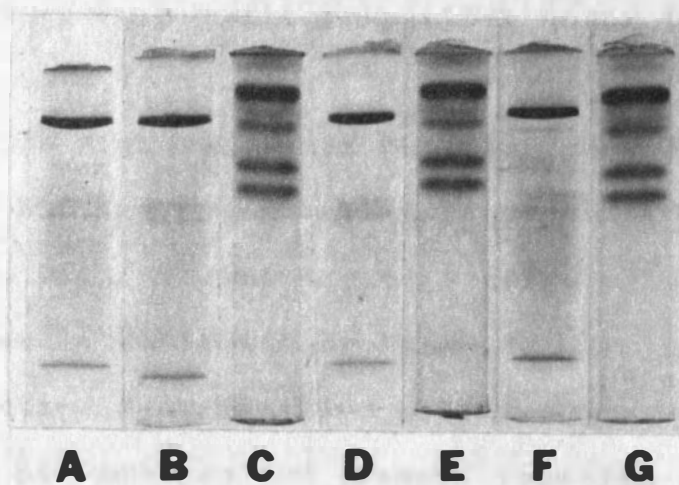


Fig. 14

CONCLUSIONS

Fraction 1 Protein

Fraction 1 protein was identified as the relatively large, dark staining band near the top of the polyacrylamide gel. This is probably a heterogeneous group of proteins with similar electrophoretic properties under these conditions.

Qualitative differences in proteins were detected among species but not among varieties of alfalfa nor between clones within a variety or species. No qualitative differences due to daylength or stage of growth were found in either Fraction 1 or Fraction 2 proteins.

Analysis of variance for percent Fraction 1 protein, measured as a percentage of the total soluble proteins, showed no statistically significant differences among varieties, stages of growth, nor between daylengths when birdsfoot trefoil and white clover were excluded from the analysis.

The percent Fraction 1 protein of birdsfoot trefoil did not differ statistically from three varieties of alfalfa. Both birdsfoot trefoil and Teton alfalfa were significantly lower than Vernal alfalfa and white clover.

Examination of the percent Fraction 1 protein variety or species means did not show differences large enough to

adequately explain the absence of bloat when birdsfoot trefoil is grazed. Some variation due to treatments is also shown but again these do not differ sufficiently to determine bloat-nonbloat conditions.

Because there are known differences in the potential bloat hazard among the legumes tested, these data indicate that ruminant bloat is probably not caused solely by the percentage of Fraction 1 protein in legumes.

Total Soluble Proteins

Although statistical differences can be shown among alfalfa varieties in the amount of total soluble protein, these differences are not large. Birdsfoot trefoil contained about half the amount of total soluble protein as the other species. White clover was lower in total soluble protein than any of the alfalfa varieties. Since Ladino clover, a form of white clover, has been observed to produce a higher incidence of bloat than alfalfa, these data will not support a hypothesis that bloat is due to the content of total soluble protein.

Lipoproteins

Lipoproteins studied by polyacrylamide gel electrophoresis show definite patterns which are peculiar to each clone within a variety or species. Birdsfoot trefoil is an exception to this.

Staining patterns do not seem to be qualitatively or quantitatively affected by the stage of growth at which the plants were harvested nor by a change in daylength.

The positive association between the amount of lipoproteins and the in vitro foaming capacity of the six clones of Vernal alfalfa is strong enough to suspect lipoproteins as being the surfactants responsible, at least in part, for the formation of stable foam during ruminant bloat. The physical properties of lipoprotein molecules suggest that they could participate in the formation of a stable foam. These data show that ruminant bloat is probably not caused by the amount of Fraction 1 protein in legumes and it has previously been shown that plant lipid content is not correlated with bloat. The hypothesis is proposed that legume lipoproteins are the surfactants responsible for the formation of a stable foam during bloat. The quantitative relationships among legume lipoproteins, foaming capacity, and bloat need to be studied to test this hypothesis.

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